(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 August 2003 (07.08.2003)

PCT

(10) International Publication Number
WO 03/063903 A2

(51) International Patent Classification[7]: A61K 45/00

(21) International Application Number: PCT/EP03/01044

(22) International Filing Date: 3 February 2003 (03.02.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/319,108 1 February 2002 (01.02.2002) US

(71) Applicant *(for all designated States except US)*: PRO-BIODRUG AG [DE/DE]; Weinbergweg 22 / Biozentrum, 06120 Halle/Saale (DE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: HOFFMANN, Torsten [DE/DE]; Weinbergweg 22 / Biozentrum, 06120 Halle/Saale (DE). HILDEBRANDT, Martin [DE/DE]; Weinbergweg 22 / Biozentrum, 06120 Halle/Saale (DE). DEMUTH, Hans-Ulrich [DE/DE]; Weinbergweg 22 / Biozentrum, 06120 Halle/Saale (DE).

(74) Agents: FORSTMEYER, Dietmar et al.; Boeters & Bauer, Bereiteranger 15, 81541 München (DE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**
— *without international search report and to be republished upon receipt of that report*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 03/063903 A2

(54) Title: MODULATION OF T LYMPHOCYTES USING DP IV INHIBITORS

(57) Abstract: The present invention discloses a method for therapeutically treating mammals, including but not limited to humans, to modulate immune responses via modulation of surface expression of co-stimulatory antigens on T lymphocytes. oral administration of a DP IV inhibitor causes a potent immunosuppressive effect. This immunosuppressive effect of DP IV inhibitors is exerted by an enhancement of CTLA-4 surface expression, with simultaneous inhibition of CD28 surface expression, on T cells. Furthermore, the present invention discloses preparation of a medicament, comprising one or more inhibitors of dipeptidyl peptidase enzymatic activity, for the treatment of immune disorders such as allograft rejection or autoimmune disease.

# Modulation of T Lymphocytes Using DP IV Inhibitors

## Background of Invention

### Field of the Invention

The present application relates to the function of dipeptidyl peptidase IV (DP IV, synonym: DPP IV, CD26, EC 3.4.14.5) and DP IV-like enzymes within a subject and their biological effects on the modulation of immune responses via modulation of surface expression of costimulatory antigens on T lymphocytes.The application relates further to the treatment of immune disorders such as allograft rejection or autoimmune disease by selective modulation of the activity of DP IV-like enzymes due to the use inhibitors of dipeptidyl peptidase IV activity in pharmacological doses.

### Background

Dipeptidyl peptidase IV (DP IV) is a serine protease which cleaves off N-terminal dipeptides from a peptide chain containing, preferably a proline residue in the penultimate position.

DP IV-like enzymes are structurally related enzymes to DP IV such as Dipeptidyl Peptidase II and Dipeptidyl Peptidase ß (Blanco et. al., 1998) which may share a certain sequence homology to the DP IV-CD26 sequence, but which share even if they are not structurally related (by convergent evolution) the substrate specificity of DP IV (CD26) of removing dipeptides from the N-termini of polypeptides by cleaving after a penultimate proline residue. Such enzymes including DP IV, DP II at one hand and

attractin at the other (Fukasawa et al., 2001) are also capable to remove dipeptides with a penultimate alanine (or serine or glycine residues) from the N-termini of polypeptides but usually with reduced catalytic efficacy as compared to the post-proline cleavage (Yaron & Naider, 1993).

More recently, it was discovered, that DP IV is responsible for cleaving glucagon-like peptide-1 and gastric inhibitory peptides, thereby shortening the half life of GLP-1 and GIP and their physiological response in the circulation. From inhibition of serum DP IV, a significant increase in the bioactivity of the incretins is anticipated. Since the incretins are major stimulators of pancreatic insulin secretion and have direct beneficial effects on glucose disposal, DP IV inhibition appears to represent an attractive approach for treating impaired glucose tolerance and non-insulin-dependent diabetes mellitus (NIDDM) and related disorders, like glucosuria and metabolic acidosis (see DE 196 16 486 and WO 97/40832).

With respect to immune system related function of dipeptidyl peptidase IV, it was shown, that combinations of inhibitors of the enzyme activities of dipeptidyl peptidase IV and alanyl aminopeptidase or angiotensin converting enzyme or prolyl oligopeptidase or X-pro aminopeptidase inhibit the DNA synthesis and the proliferation of mononuclear cells and T-cells (see DE 100 25 464 and WO 01/89569). Dipeptidyl peptidase IV (DP IV, CD26) has multiple functions, including cell adhesion, cellular trafficking through the extracellular matrix and co-stimulatory potential

during T cell activation. By virtue of its enzymatic activity, DP IV is capable of expanding a T cell proliferative response *in vitro* (Tanaka et al). Potent cytokines such as RANTES (Oravecz et al), SDF-1 alpha (Proost et al), MCP-2 (Oravecz et al) and TNF-alpha (Bauvois et al) are among the substrates for DP IV described to date, suggesting a complex immunomodulatory role for DP IV enzymatic activity. The T cell activation antigen CD26 possesses DP IV activity. CD26 is expressed on distinct subsets of human immune cells, as well as on murine splenocytes and thymocytes (Vivier et al). Surface expression of CD 26 is upregulated upon mitogenic or antigenic activation *in vitro* (Mattern et al, Yamabe et al). High expression of CD26 (DP IV) has been demonstrated to define a Th1/Th0 phenotype among T lymphocytes with an enhanced production of Th1-like cytokines, namely γ - interferon (Willheim et al).

Borloo & De Meester, 1994, proposed 5 types of potential DP IV inhibitors: azapeptides, azetidines, Michael substrates, reduced peptides and phosphonic acids. All the synthesized compounds possess a substrate-like structure, which is a pre-requisite for recognition by the enzyme. A modified proline or alanine at the penultimate position was choosen, substituted with glycine, alanine, valine, isoleucine or phenylalanine at the N-terminus. The prepared compounds were screened *in vitro* at a 5 mM concentration with a fluorometric method using Gly-Pro-4-Me-2NA as substrate.

Examples of low molecular weight dipeptidyl

peptidase IV inhibitors are agents such as, tetrahydroisoquinolin-3-carboxamide derivatives, N-substituted 2-cyanopyroles and pyrrolidines, N-(N"-substituted glycyl)2-cyanopyrrolidines, N-(substituted glycyl)-thiazolidines, N-(substituted glycyl)-4-cyanothiazolidines, L- *threo* -isoleucyl thiazolidine, L- *allo* -isoleucyl thiazolidine, L- *threo* -isoleucyl pyrrolidine, L- *allo* -isoleucyl thiazolidine, L- *allo* -isoleucyl pyrrolidine, boronyl inhibitors and cyclopropyl-fused pyrrolidines. Inhibitors of dipeptidyl peptidase IV are described in US 6,011,155; US 6,107,317; US 6,110,949; US 6,124,305; US 6,172,081; WO 99/61431, WO 99/67278, WO 99/67279, DE 198 34 591, WO 97/40832, DE 196 16 486 C 2, WO 98/19998, WO 00/07617, WO 99/38501, WO 99/46272, WO 99/38501, WO 01/68603, WO 01/40180, WO 01/81337, WO 01/81304 and WO 01/55105, the teachings of which are herein incorporated by reference in their entirety.

**Summary of Invention**

This patent application addresses the therapeutic use of DP IV inhibitors for the modulation of immune responses via modulation of surface expression of co-stimulatory antigens on T lymphocytes. Based on studies in two different mouse models of immune responses, evidence is provided for a hitherto unknown effect of inhibitors of the enzyme Dipeptidyl peptidase IV (DP IV, CD26), on the surface expression of T cell antigens with co-stimulatory activity, CD28 and CTLA-4. Surprisingly, as one embodiment of this application it was found, that the potent immunosuppressive effect of DP IV inhibitors is exerted by an enhancement of CTLA-4

surface expression, with simultaneous inhibition of CD28 surface expression, on T cells. These novel findings pave the way for a therapeutic use of DP IV inhibitors in immune disorders such as allograft rejection or autoimmune disease.

**Brief Description of Drawings**

Figure 1: CTLA-4 surface expression on CD26-positive decidual lymphocytes

Figure 2: CTLA-4 surface expression on CD26-positive splenocytes (MFI+/- SD)

Figure 3: Box plots for the mean flurescence intensity (MFI) of CD28 on CD4-positive splenocytes

Figure 4: Box plots for the mean flurescence intensity (MFI) of CTLA-4 on CD4-positive splenocytes

**Detailed Description**

In immune responses, T cells are activated following presentation of a specific antigen. The process of T cell activation by specific cells capable of antigen presentation has been elaborated in detail (for review cf. Guinan et al; Salomon B & Bluestone JA). Three major steps can be segregated in this process: 1) the adhesion of the T cell and the Antigen-Presenting Cell (APC) via adhesion molecules, 2) the recognition of a distinct antigen presented via an MHC (major histocompatibility) antigen, a process that induces an initial increase in the production of cytokines such as interleukin-2 acting on the T cell in an autocrine loop, and 3) the binding of co-stimulatory T cell antigens to specific ligands on the surface of the antigen-presenting cell. It appears that the third

step, i.e. the binding of the co-stimulatory antigen to the corresponding ligand, is essential for a full T cell activation as a central component of an antigen-specific immune response. Several pairs of receptors and respective ligands are known for all of these three steps. Among the co-stimulatory antigens, i.e. the components of step three of the T cell activation cascade, the antigens CD28 and CTLA-4 have raised substantial interest in recent years. Both share a high degree of structural similarity, and both bind to the same ligands expressed on the surface of antigen-presenting cells, CD80 and CD86 (B7-1 and B7-2).

Studies on the mechanism by which CD28 and CTLA-4 contribute to T cell activation have revealed that CTLA-4 competes with CD28 for binding to their respective ligands, B7-1 and B7-2. Even more so, CTLA-4, expressed upon T cell activation, induces a state of anergy in the T cell upon binding to its ligand, thus limiting the activation process and resulting in immune tolerance in vivo, whereas binding of CD28 to the same ligand provides the decisive signal for full-blown T cell activation. This has been conclusively shown by the use of antibodies blocking CD28 (Alegre et al), or by soluble CTLA-4/immunoglobulin fusion proteins interacting with the T cell activation process (Lee RS et al). Both competitors, CD28 and CTLA-4, may be expressed at the same time, with the amount of CD28- and CTLA-4-molecules present on the cell surface determining the outcome of the process of T cell activation. The data known so far lead to the appraisal of the competitive action of CD28 and CTLA-4 as a

dynamic process that may initiate as well as limitate T cell activation and thus control the extent of an immune response (Chambers et al).

The knowledge on CD28 and CTLA-4 yields to a potential therapeutic use for the modulation of immune responses. In fact, immunosuppressants used in various conditions, such as Cyclosporin A and tacrolimus (FK-506), apparently do not interact with T cell costimulation modulated by CD28 and CTLA-4.

To date, no link between DP IV activity and surface expression of the co-stimulatory antigens CD28 and CTLA-4 has been established. It is the very preferred embodiment of the present application to use inhibitors of DP IV activity to enhance surface expression of CTLA-4 while limiting the surface expression of CD28 T cells of a subject.

The present invention especially relates to the use of a DP IV activity lowering effector for the preparation of a medicament for the change of the surface expression of co-stimulatory antigens on T-lymphocytes in an animal. According to one preferred embodiment it refers to the use of a DP IV activity lowering effector for the change of the surface expression of co-stimulatory antigens on T-lymphocytes in an animal, comprising administering to said animal a therapeutically effective dose of at least one DP IV activity lowering effector.

The term "subject"as used herein, refers to an animal, preferably a mammal, most preferably a human, who has been the object of treatment, observation or

experiment.

The "DP IV inhibitor", as used herein, may be agents such as, tetrahydroisoquinolin-3-carboxamide derivatives, N-substituted 2-cyanopyroles and pyrrolidines, N-(N"-substituted glycyl)2-cyanopyrrolidines, N-(substituted glycyl)-thiazolidines, N-(substituted glycyl)-4-cyanothiazolidines, L- *threo* -isoleucyl thiazolidine, L- *allo* -isoleucyl thiazolidine, L- *threo* -isoleucyl pyrrolidine, L- *allo* -isoleucyl thiazolidine, L- *allo* -isoleucyl pyrrolidine, boronyl inhibitors and cyclopropyl-fused pyrrolidines. Inhibitors of dipeptidyl peptidase IV are described in US 6,011,155; US 6,107,317; US 6,110,949; US 6,124,305; US 6,172,081; WO 99/61431, WO 99/67278, WO 99/67279, DE 198 34 591, WO 97/40832, DE 196 16 486 C 2, WO 98/19998, WO 00/07617, WO 99/38501, WO 99/46272, WO 99/38501, WO 01/68603, WO 01/40180, WO 01/81337, WO 01/81304 and WO 01/55105, the teachings of which are herein incorporated by reference in their entirety.

The term "therapeutically effective amount" as used herein, means that amount of active compound or pharmaceutical agent that elicits the biological or medicinal response in a tissue system, animal or human, being sought by a researcher, veterinarian, medical doctor or other clinician, which includes alleviation of the symptoms of the disease or disorder being treated.

As used herein, the term "composition" is intended to encompass a product comprising the claimed inhibitors of DP IV activity in the therapeutically

effective amounts, as well as any product which results, directly or indirectly, from combinations of the claimed compounds.

In another preferred embodiment of the present invention, the inhibitors of the DP IV activity may also be present in the form of a pharmaceutically acceptable salt.

The present invention further includes within its scope prodrugs of the DP IV inhibitors. In general, such prodrugs will be functional derivatives of the compounds which are readily convertible *in vivo* into the desired therapeutically active compound. Thus, in these cases, the methods of treatment of the present invention, the term "administering" shall encompass the treatment of the various disorders described with prodrug versions of one or more of the claimed compounds, but which converts to the above specified compound *in vivo* after administration to the subject. Conventional procedures for the selection and preparation of suitable prodrug derivatives are described, for example, in "Design of Prodrugs", ed. H. Bundgaard, Elsevier, 1985 and the patent applications WO 99/67278 and WO 99/67279, fully incorporated herein by reference.

Where the compounds according to this invention have at least one chiral center, they may accordingly exist as enantiomers. Where the compounds possess two or more chiral centers, they may additionally exist as diastereomers. It is to be understood that all such isomers and mixtures thereof are encompassed within the scope of the present invention. Furthermore, some of

the crystalline forms of the compounds may exist as polymorphs and as such are intended to be included in the present invention. In addition, some of the compounds may form solvates with water (i.e. hydrates) or common organic solvents, and such solvates are also intended to be encompassed within the scope of this invention.

The modulators of this invention may be prepared using solid phase chemistry or, alternatively, via normal solution chemistry, using conventional methods of known in the art.

Another preferred embodiement of the present invention is the use of one or more DP IV inhibitor for the preparation of a medicament for the treatment of a condition mediated by modulation of the DP IV activity in a subject. The compound maybe administered to a patient by any conventional route of administration, including, but not limited to, intravenous, oral, subcutaneous, intramuscular, intradermal, intranasal and parenteral.

The present invention also provides pharmaceutical compositions comprising one or more DP IV inhibitors in association with a pharmaceutically acceptable carrier.

To prepare the pharmaceutical compositions of this invention, one or more DP IV inhibitors or salts thereof is intimately admixed with a pharmaceutical carrier according to conventional pharmaceutical compounding techniques, which carrier may take a wide variety of forms depending of the form of preparation desired for administration, e.g., oral or parenteral

such as intramuscular. In preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed. Thus, for liquid oral preparations, such as for example, suspensions, elixirs and solutions, suitable carriers and additives may advantageously include water, glycols, oils, alcohols, flavoring agents, preservatives, coloring agents and the like; for solid oral preparations such as, for example, powders, capsules, gelcaps and tablets, suitable carriers and additives include starches, sugars, diluents, granulating agents, lubricants, binders, disintegrating agents and the like. Because of their ease in administration, tablets and capsules represent the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are employed. If desired, tablets may be sugar coated or enteric coated by standard techniques. For parenterals, the carrier will usually comprise sterile water, through other ingredients, for example, for purposes such as aiding solubility or for preservation, may be included.

Injectable suspensions may also prepared, in which case appropriate liquid carriers, suspending agents and the like may be employed. The pharmaceutical compositions herein will contain, per dosage unit, e.g., tablet, capsule, powder, injection, teaspoonful and the like, an amount of the active ingredient necessary to deliver an effective dose as described above. The pharmaceutical compositions herein will contain, per unit dosage unit, e.g., tablet, capsule, powder, injection, suppository, teaspoonful and the

like, of from about 0.03 mg to 100 mg/kg (preferably 0.1 30 mg/kg) and may be given at a dosage of from about 0.1 300 mg/kg/day (preferably 1 50 mg/kg/day). The dosages, however, may be varied depending upon the requirement of the patients, the severity of the condition being treated and the compound being employed. The use of either daily administration or post-periodic dosing may be employed. Typically the dosage will be regulated by the physician based on the characteristics of the patient, his/her condition and the therapeutic effect desired.

Preferably these compositions are in unit dosage forms from such as tablets, pills, capsules, powders, granules, sterile parenteral solutions or suspensions, metered aerosol or liquid sprays, drops, ampoules, autoinjector devices or suppositories; for oral parenteral, intranasal, sublingual or rectal administration, or for administration by inhalation or insufflation. Alternatively, the composition may be presented in a form suitable for once-weekly or once-monthly administration; for example, an insoluble salt of the active compound, such as the decanoate salt, may be adapted to provide a depot preparation for intramuscular injection. For preparing solid compositions such as tablets, the principal active ingredient is ideally mixed with a pharmaceutical carrier, e.g. conventional tableting ingredients such as corn starch, lactose, sucrose, sorbitol, talc, stearic acid, magnesium stearate, dicalcium phosphate or gums, and other pharmaceutical diluents, e.g. water, to form a solid preformulation composition containing a

homogeneous mixture of a compound of the present invention, or a pharmaceutically acceptable salt thereof. When referring to these preformulation compositions as homogeneous, it is meant that the active ingredient is ideally dispersed evenly throughout the composition so that the composition may be readily subdivided into equally effective dosage forms such as tablets, pills and capsules. This solid preformulation composition may then be subdivided into unit dosage forms of the type described above containing from 0.1 to about 500 mg of the active ingredient of the present invention.

The tablets or pills of the novel composition can be advantageously coated or otherwise compounded to provide a dosage form affording the advantage of prolonged action. For example, the tablet or pill can comprise an inner dosage and an outer dosage component, the latter being in the form of an envelope over the former. The two components can be separated by an enteric layer which serves to resist disintegration in the stomach and permits the inner component to pass intact into the duodenum or to be delayed in release. A variety of materials can be used for such enteric layers or coatings, such materials including a number of polymeric acids with such materials as shellac, cetyl alcohol and cellulose acetate.

This liquid forms in which the novel compositions of the present invention may be advantageously incorporated for administration orally or by injection include, aqueous solutions, suitably flavoured syrups, aqueous or oil suspensions, and flavoured emulsions

with edible oils such as cottonseed oil, sesame oil, coconut oil or peanut oil, as well as elixirs and similar pharmaceutical vehicles. Suitable dispersing or suspending agents for aqueous suspensions include synthetic and natural gums such as tragacanth, acacia, alginate, dextran, sodium carboxymethylcellulose, methylcellulose, polyvinylpyrrolidone or gelatin.

Where the processes for the preparation of the compounds according to the invention give rise to mixture of stereoisomers, these isomers may be separated by conventional techniques such as preparative chromatography. The compounds may be prepared in racemic form, or individual enantiomers may be prepared either by enantiospecific synthesis or by resolution. The compounds may, for example, be resolved into their components enantiomers by standard techniques, such as the formation of diastereomeric pairs by salt formation with an optically active acid, such as (-)-di-p-toluoyl-d-tartaric acid and/or (+)-di-p-toluoyl-l-tartaric acid followed by fractional crystallization and regeneration of the free base. The compounds may also resolved by formation of diastereomeric esters or amides, followed by chromatographic separation and removal of the chiral auxiliary. Alternatively, the compounds may be resolved using a chiral HPLC column.

During any of the processes for preparation of the compounds of the present invention, it may be necessary and/or desirable to protect sensitive or reactive groups on any of the molecules concerned. This may be achieved by means of conventional protecting

groups, such as those described in Protective Groups in Organic Chemistry, ed. J.F.W. McOmie, Plenum Press, 1973; and T.W. Greene & P.G.M. Wuts, Protective Groups in Organic Synthesis, John Wiley & Sons, 1991, fully incorporated herein by reference. The protecting groups may be removed at a convenient subsequent stage using methods known from the art.

The method of treating conditions modulated by dipetidyl peptidase IV and DP IV - like enzymes described in the present invention may also be carried out using a pharmaceutical composition comprising any of the compounds as defined herein and a pharmaceutically acceptable carrier. The pharmaceutical composition may contain between about 0.01 mg and 100 mg, preferably about 5 to 50 mg, of the compound, and may be constituted into any form suitable for the mode of administration selected. Carriers include necessary and inert pharmaceutical excipients, including, but not limited to, binders, suspending agents, lubricants, flavorants, sweeteners, preservatives, dyes, and coatings. Compositions suitable for oral administration include solid forms, such as pills, tablets, caplets, capsules (each including immediate release, timed release and sustained release formulations), granules, and powders, and liquid forms, such as solutions, syrups, elixirs, emulsions, and suspensions. Forms useful for parenteral administration include sterile solutions, emulsions and suspensions.

Advantageously, compounds of the present invention may be administered in a single daily dose, or the total daily dosage may be administered in

divided doses of two, three or four times daily. Furthermore, compounds for the present invention can be administered in intranasal form via topical use of suitable intranasal vehicles, or via transdermal skin patches well known to those of ordinary skill in that art. To be administered in the form of transdermal delivery system, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen and dosage strength will need to be accordingly modified to obtain the desired therapeutic effects.

For instance, for oral administration in the form of a tablet or capsule, the active drug component can be combined with an oral, non-toxic pharmaceutically acceptable inert carrier such as ethanol, glycerol, water and the like. Moreover, when desired or necessary, suitable binders; lubricants, disintegrating agents and coloring agents can also be incorporated into the mixture. Suitable binders include, without limitation, starch, gelatin, natural sugars such as glucose or betalactose, corn sweeteners, natural and synthetic gums such as acacia, tragacanth or sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, sodium chloride and the like. Disintegrators include, without limitation, starch, methyl cellulose, agar, bentonite, xanthan gum and the like.

The liquid forms in suitable flavored suspending or dispersing agents such as the synthetic and natural gums, for example, tragacanth, acacia, methyl-cellulose and the like. For parenteral administration, sterile

suspensions and solutions are desired. Isotonic preparations which generally contain suitable preservatives are employed when intravenous administration is desired.

The compound of the present invention can also be administered in the form of liposome delivery systems, such as small unilamellar vesicles, large unilamellar vesicles, and multilamellar vesicles. Liposomes can be formed from a variety of phospholipids, such as cholesterol, stearylamine or phosphatidylcholines using processes well described in the art.

Compounds of the present invention may also be delivered by the use of antibodies, most preferably monoclonal antibodies as individual carriers to which the compound molecules are coupled. The compounds of the present invention may also be coupled with soluble polymers as targetable drug carriers. Such polymers can include polyvinylpyrrolidone, pyran copolymer, polyhydroxypropylmethacrylamidephenol, polyhydroxyethylaspartamidephenol, or polyethyl eneoxidepolyllysine substituted with palmitoyl residue. Furthermore, the compounds of the present invention may be coupled to a class of biodegradable polymers useful in achieving controlled release of a drug, for example, polyactic acid, polyepsilon caprolactone, polyhydroxy butyeric acid, polyorthoesters, polyacetals, polydihydropyrans, polycyanoacrylates and cross-linked or amphipathic block copolymers of hydrogels.

DP IV inhibitors according to the present invention may be administered in any of the foregoing compositions and according to dosage regimens

established in the art whenever treatment of the addressed disorders is required.

The daily dosage of the products may be varied over a wide range from 0.01 to 1.000 mg per adult human per day. For oral administration, the compositions are preferably provided in the form of tablets containing, 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100, 150, 200, 250 and 500 milligrams of the active ingredient for the symptomatic adjustment of the dosage to the patient to be treated. An effective amount of the drug is ordinarily supplied at a dosage level of from about 0.1 mg/kg to about 300 mg/kg of body weight per day. Preferably, the range is from about 1 to about 50 mg/kg of body weight per day. The compounds may be administered on a regimen of 1 to 4 times per day.

Optimal dosages to be administered may be readily determined by those skilled in the art, and will vary with the particular compound used, the mode of administration, the strength of the preparation, bioavailability due to the mode of administration, and the advancement of disease condition. In addition, factors associated with the particular patient being treated, including patient age, weight, diet and time of administration, should generally be considered in adjusting dosages.

To prepare the pharmaceutical compositions according to the present invention, one or more DP IV inhibitors or salts thereof of the invention as the active ingredient, is ideally intimately admixed with a pharmaceutical carrier according to conventional

pharmaceutical compounding techniques, which carrier may take a wide variety of forms depending of the form of preparation desired for administration, e.g., oral or parenteral such as intramuscular. In preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed. Thus, for liquid oral preparations, such as for example, suspensions, elixirs and solutions, suitable carriers and additives include water, glycols, oils, alcohols, flavoring agents, preservatives, coloring agents and the like; for solid oral preparations such as, for example, powders, capsules, caplets, gelcaps and tablets, suitable carriers and additives include starches, sugars, diluents, granulating agents, lubricants, binders, disintegrating agents and the like. Because of their ease in administration, tablets and capsules represent the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are obviously employed. If desired, tablets may be sugar coated or enteric coated by standard techniques. For parenterals, the carrier will usually comprise sterile water, through other ingredients, for example, for purposes such as aiding solubility or for preservation, may be included. Injectable suspensions may also prepared, in which case appropriate liquid carriers, suspending agents and the like may be employed. The pharmaceutical compositions herein will contain, per dosage unit, e.g., tablet, capsule, powder, injection, teaspoonful and the like, an amount of the active ingredient necessary to deliver an effective dose as described above. The pharmaceutical compositions herein

will contain, per unit dosage unit, e.g., tablet, capsule, powder, injection, suppository, teaspoonful and the like, of from about 0.03 mg to 100 mg/kg (preferred 0.1 30 mg/kg) and may be given at a dosage of from about 0.1 300 mg/kg/day (preferred 1 50 mg/kg/day). The dosages, however, may be varied depending upon the requirement of the patients, the severity of the condition being treated and the compound being employed. The use of either daily administration or post-periodic dosing may be employed.

**Examples**

*Example 1: stress-induced abortions in mice*

The study relies on a murine model of stress-induced, cytokine-mediated abortions as described previously (Arck et al; Hildebrandt et al). Beyond aspects related to biology of reproduction, this model represents an attractive approach for the study of immune mechanisms and stress-related immune alterations *in vivo* . It offers the advantages of a clearly defined starting point (pregnancy), a precise endpoint (abortion) and a simple read-out (percentage of abortions among implants). Furthermore, given the average number of implants in murine pregnancy of 8-9 implants per animal, the overall number of animals needed for a reliable analysis and interpretation of data is relatively low when compared to other animal models for the study of immune alterations and disorders.

*Rationale of study design*

In murine pregnancy, the decisive shift from a pregnancy-protective Th2 cytokine response towards a

pro-inflammatory Th1 cytokine pattern is able to exert its abortogenic effect in the peri-implantation period between day 5.5 and 8.5 of murine pregnancy (Clark et al). With regard to abortions, the DBA-mated CBA mice used in our study were shown to be sensitive to a defined stressor on day 5.5 of pregnancy (Arck et al). For this reason, the application of the DP IV inhibitor ilsoleucyl-cyanopyrrolidine (Ile-Pyr) was started on day 5.5 immediately prior to exposure to the stressor in the respective group of animals and continued until day 13.5 when the animals were sacrificed.

*Animals*

Female CBA/J and male DBA/2 mice were purchased from Charles River, Germany, and maintained in an animal facility with a 12 hour light/dark cycle. After overnight cohabitation of CBA/J females with a DBA/2 male, females with vaginal plugs (day 0.5 of pregnancy) were segregated. Subgroups of at least 20 mated mice received daily intraperitoneal injections of ilsoleucyl-cyanopyrrolidine (0,5 µ mol/kg/d in 0.2 ml PBS) starting on day 5.5 of pregnancy. Injection of the inactive stereoisomeric form of ilsoleucyl-cyanopyrrolidine was used as a control. The groups were divided, and half of the animals receiving ilsoleucyl-cyanopyrrolidine or the control substance, respectively, were expos7ed to ultrasonic stress for 24 hours beginning on day 5.5 to boost abortion rates as described previously (Arck et al). All animals were sacrificed on day 13.5, the numbers of normal and resorbing sites were determined, and cells were taken for further study.

*Preparation of cell suspensions and flow cytometry:*

Mononuclear cells were prepared from murine spleens and deciduae as described (Arck et al). All monoclonal antibodies (mAb) were purchased from Pharmingen (Heidelberg, Germany) and included the following: R-Phycoerythrin-labeled (R-PE) hamster anti-mouse mAb against CD3 (clone G4.18), CD69 (clone H1.2F3), CD4 (clone GK1.5), CD28 (clone 37.51); Fluorescein Isothiocyanate (FITC)-conjugated rat anti-mouse mAb against CD4 (clone RM 4-5), CD26 (clone H194-112); and Allophycocyanin (APC)-conjugated rat anti-mouse against CD8a (clone 53-6.7). Unconjugated hamster anti-mouse CTLA-4/CD152 (clone UC10-4F10-11) was used. Each antibody was diluted 1:100. All incubations were performed for 20 minutes at 20 ° C in the dark. First, the cell suspensions ($0.5-1x10^6$ cells)were incubated with FITC-conjugated mAbs. After washing, PE-, APC-conjugated mAbs and the unconjugated CTLA-4/CD152-mAb were added to six of the seven suspensions, respectively. For intracellular staining of CTLA-4, one aliquot received 100 $\mu$ l of permeabilization buffer before incubation with CTLA-4 mAb dissolved in permeabilization buffer. Cell suspensions were incubated again. Finally, unconjugated CTLA-4 mAb was labelled with biotin-conjugated mouse anti-hamster IgG mAb cocktail (clones G70-204 and G94-56) and Streptavidin-Phycoerythrin (PE) (Pharmingen, Heidelberg, Germany).

Cell fluorescence was measured using a Becton Dickinson FACSCalibur Flow Cytometer. A gate for the

lymphocyte population was defined by forward and side light scatter characteristics (size/complexity criteria). Gating of CD3-, CD4- and CD8-positive cell populations was performed based on a scatter plot showing side scatter and the respective fluorescence characteristics. Negative control samples incubated with irrelevant fluorochrome-conjugated, isotype-matched antibodies were performed in parallel and generated < 0.3 % fluorescent cells.

The results of the cytofluorometric analyses were recorded

- as the percentage number of cells positive for the respective antibody or, in double-staining techniques, for two different antibodies, as compared to an isotype control,

- as the mean fluorescence intensity of the respective antibody-positive population.

Controls included groups of stressed and non-stressed animals. Routinely, at least two independent series of experiments were performed to obtain confirmation for the experimental results obtained in the first series.

*Results*

The use of isoleucyl-cyanopyrrolidine resulted in an abrogation of stress-induced, cytokine-mediated abortions (39,2% in stressed animals without the inhibitor isoleucyl-cyanopyrrolidine, 7,5% in stressed animals with isoleucyl-cyanopyrrolidine, $p<0.01$). We observed a reduction of the stress-induced increase of CD26-positive decidual lymphocytes upon application of isoleucyl-cyanopyrrolidine (20 vs. 41%, $p<0.05$) (see

figure 1). Furthermore, local (i.e., decidua) and systemic changes (i.e., spleen) of distinct cell populations were observed, such as the subset of cells coexpressing the antigens CD26 and CD4: this population showed a significant decrease among the splenocytes upon application of isoleucyl-cyanopyrrolidine (3,4% vs. 14% among all gated cells, $p<0.01$) (see figure 2).

Using isoleucyl-cyanopyrrolidine, modifications of the time period in which the animals were injected were explored. In addition to the established schedule of daily injections from day 5.5 until day 13.5 of murine pregnancy, additional subgroups of animals were injected daily from day 5.5 until day 8.5 and on day 5.5, i.e. immediately prior to stress exposure, only. A single injection of P 59/99 in the concentration described above (0.5 $\mu$ mol/ kg b.w.) was sufficient to induce the same immunologic changes as observed using the established pattern of injections and also to abrogate the expected increase in murine abortions (13,9% (day 5.5, n=6) und 10,2% (day 5.5 until day 13.5, n=6) vs. 36,4% (stressed animals without the inhibitor, n=10), $p<0.01$).

In stressed animals, a lower surface densitiy of CTLA-4 on decidual CD26-positive lymphocytes was observed than in non-stressed animals; inhibition of DP IV restored CTLA-4 surface densitiy to normal (94,3 vs. 30,2 arbitrary units; $p<0,01$). CD26/DPPIV-positive splenocytes showed a higher surface expression of CTLA-4 upon DP IV inhibition, irrespective of stress exposure (71,3+/-SD vs. 40,9+/-SD; $p<0,05$).

*Example 2: Injection of nonpregnant female*

*C57BL/6 mice with Mycobacterium bovis (Bacillus Calmette-Gu é rin , BCG)*

Female C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany) and maintained in an animal facility with a 12 hour light/dark cycle. Animal care and experimental procedures followed instiutional ethic guidelines and conformed to requirements of the state authority for animal research conduct (LAGetSi, Berlin). The 64 mice were divided into two groups of 32 animals each. One group received daily intraperitoneal injections of the DP-IV inhibitor Ile-Cyanopyrrolidide (0,5 µ mol/kg/d in 0.2 ml Phosphate-Buffered-Saline) for the first 3 days. Mice of the other group received daily injections of 0,2 ml Phosphate-buffered saline and served as controls. Half of the animals (n=16) of each group received an i.p.-injection of 200 µ l (=4 Mill. I.U.) of BCG i.p. while the other half received 200 µ l of PBS-buffer. 8 animals of each group were sacrificed on day 3, the other half on day 38, and the spleen and blood were taken for further study.

*Preparation of cell suspensions*

Splenocytes were harvested by passage of the spleen through a 100- µ m cell strainer (BD Falcon, USA). Mononuclear cells were purified by Lympholyte/M (Cedarlane Labs, Hornby, Canada) gradient centrifugation. Afterwards cells were washed once and resuspended in RPMI 1640 Medium (Biochrom, Berlin, Germany).

*Flow cytometry*

The cell supensions used for flow cytometry were divided into 7 aliquots containing 0.5-1.0 x $10^6$ each.

The flow cytometric staining was performed following the protocol outlined above (application 1).

*Results*

CD28-expression on CD4+ splenocytes was lowered under DP-IV-inhibition. The expression of CD28 was normal under BCG-immunization (see figure 3).CTLA-4-expression on CD4+ splenocytes was lowered under BCG-stimulation compared to controls, but enhanced expression of CTLA-4 was seen under BCG-stimulation with DP-IV-inhibition compared to BCG-stimulation without DP-IV-inhibition (see figure 4).

Claims

1.The use of a DP IV activity lowering effector for the preparation of a medicament for the change of the surface expression of co-stimulatory antigens on T-lymphocytes in an animal.

2.The use according to claim 1 wherein said change causes the modulation of the immune responses in said animal.

3.The use according to claims 1 or 2 wherein said change causes the enhancement of CTLA-4 expression and the inhibition of the CD28-expression on T-lymphocytes.

4.The use according to any one of the preceding claims wherein said effector comprises a substrate which is capable of binding with said DP IV and which competes with naturally occurring substrates for DP IV.

5.The use according to any one of the preceding claims for oral application.

6.The use according to any one of the preceding claims for chronic oral application.

7.The use according to any one of the preceding claims for iv or im or icv application.

8.The use according to any one of the preceding claims 1 - 5 or 7 for chronic *iv, im* or *icv* application.

9.The use to any one of the preceding claims wherein one or more inhibitors of dipeptidyl peptidase activity are used in combination with a pharmaceutically acceptable carrier.

10. The use according to any one of the preceding claims, wherein the medicament is used for the prevention or treatment of immunological disorders.

11. The use according to any one of the preceding claims, wherein the medicament is used for the prevention or treatment of autoimmune diseases, allograft rejection and/or graft versus host rejection.

12. The use according to any one of the preceding claims 1 - 10, wherein the medicament is used for the prevention or treatment of stress induced abort in a mammal.

**Figure 1: CTLA-4 surface expression on CD26-positive decidual lymphocytes**

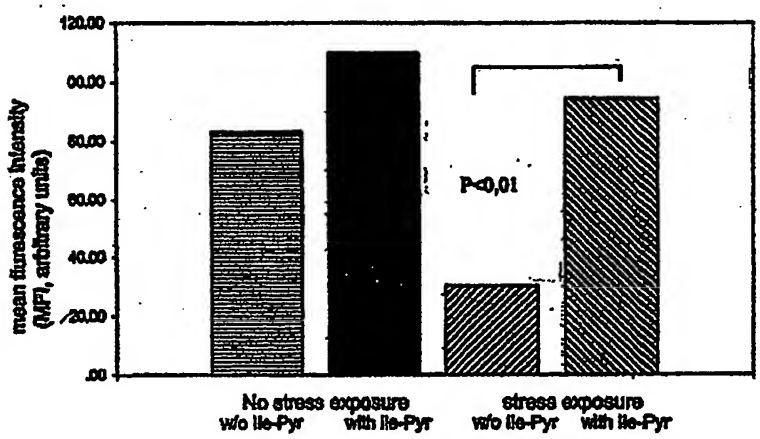

**Figure 2: CTLA-4 surface expression on CD26-positive splenocytes (MFI+/- SD)**

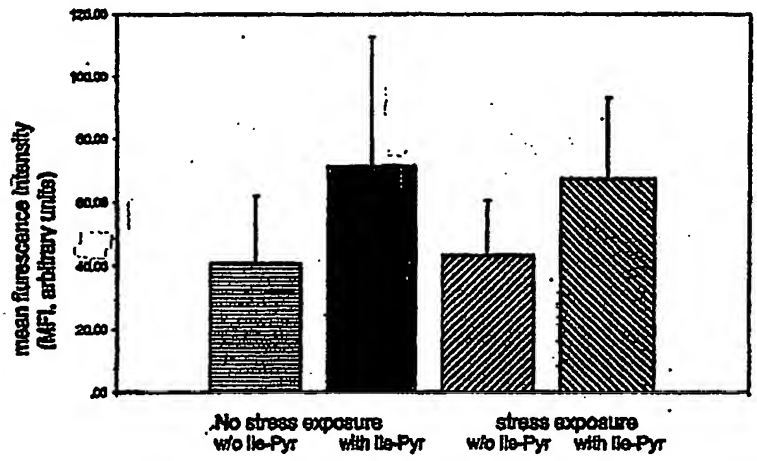

**Figure 3: Box plots for the mean fluorescence intensity (MFI) of CD28 on CD4-positive splenocytes**

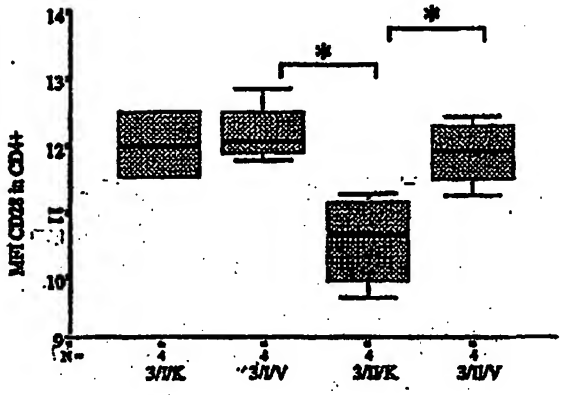

**Figure 4: Box plots for the mean flurescence intensity (MFI) of CTLA-4 on CD4-positive splenocytes**